บทความวิจัย

การชักนำให้ดีเอ็นเอกลายพันธุ์โดยเทคนิคพลาสมาพลังงานต่ำแบบความดันบรรยากาศ

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บทคัดย่อ

การศึกษาครั้งนี้มีวัตถุประสงค์เพื่อศึกษาผลของพลาสมาแบบความดันบรรยากาศ (APPJ) ต่อการชักนำให้ดีเอ็นเอเปลือยเกิดการกลายพันธุ์ ดีเอ็นเอเปลือยที่ใช้ในการทดลอง คือ พลาสมิดดีเอ็นเอที่มีโปรตีนเรืองแสงสีเขียว (pGFP) โดย pGFP ถูกระดมยิงด้วยฮีเลียมพลาสมา (He-plasma) และอาร์กอนพลาสมา (Ar-plasma) ที่พลังงาน 100 วัตต์ เป็นเวลา 10 และ 15 นาที จากนั้นส่งถ่าย พลาสมิด pGFP ที่ถูกระดมยิงเข้าสู่เซลล์แบคทีเรีย *Escherichia coli* สายพันธุ์ DH5α เพื่อตรวจสอบการกลายพันธุ์ภายใต้แสง UV โคโลนีสีขาวคือแบคทีเรียพันธุ์กลาย ในการทดลองนี้พบการกลายพันธุ์ของ pGFP เมื่อพลาสมิดดีเอ็นเอเปลือยถูกระดมยิงด้วยอาร์กอนพลาสมาที่เวลา 15 นาที เท่านั้น จากผลการวิเคราะห์ลำดับนิวคลีโอไทด์ พบการกลายพันธุ์โดยการเพิ่มขึ้นของนิวคลีโอไทด์ (insertion) และการแทนที่คู่เบส (substitution) มากที่สุด ผลจากการศึกษาครั้งนี้สามารถนำไปใช้เป็นข้อมูลพื้นฐานสำหรับการชักนำการกลายพันธุ์ของดีเอ็นเอเปลือยโดยพลาสมาแบบความดันบรรยากาศ

คำสำคัญ: อาร์กอนพลาสมา พลาสมาแบบความดันบรรยากาศ (APPJ) ฮีเลียมพลาสมา การกลายพันธุ์ ดีเอ็นเอเปลือย

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Induced Mutations of Naked DNA by Atmospheric Pressure Plasma Jet (APPJ)

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ABSTRACT

This study was aimed to investigate the effect of atmospheric pressure plasma jet (APPJ) on induced mutation to naked DNA. The DNA sample was plasmid DNA containing green fluorescent protein (pGFP). Helium (He) and argon (Ar) plasmas were chosen to bombard pGFP with energy of 100 watts for 10 and 15 min. Consequently the bombarded pGFP was transferred into *E. coli* DH5α. Then, transformed *E. coli* was screened under UV light. White colonies indicated as bacterial mutants. In this work, the mutation was observed when the naked DNA was bombarded only at 15 min with Ar plasma. DNA sequencing revealed that substitution and insertion mutations were major types of DNA mutation. The results from this study could be used as basic data for induced mutations of naked DNA by APPJ.

Keywords: Argon plasma, Atmospheric pressure plasma jet (APPJ), Helium plasma, Mutation, Naked DNA

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Introduction

Since the discovery of X-ray-induced genomic mutation in 1927 by Muller [1], varieties of mutagenic agents have been documented including X-rays, gamma rays, neutrons, UV light, chemical mutagens, high energy ion beam bombardment [2], and recent plasma immersion (under vacuum condition) [3]. Generally, plasma-based ion implantation technology at relatively lower energy has been widely applied in a variety of materials processing including surface pretreatment, deposition of films and surfaces coating in order to change the chemical composition or crystallinity of the coating [4]. Recently, APPJ with a very low energy have received great attention due to their remarkable abilities in various biomedical applications such as sterilization of both biotic and abiotic surfaces [5], blood coagulation for wound healing and surgery [6], tissue scaffold treatment [7], and killing cancer cells by apoptosis induction [8].

It has been purposed that ion beam bombardment with a very high energy in order of 100 keV has effects on DNA structure with both direct and indirect interactions [2]. However, there is no evidence on the interaction of atmospheric plasma ions (with low energy in order a few eV) on naked DNA. In order to study this, we introduced plasma ions to interact with naked or plasmid DNA at atmospheric condition to investigate relevant effects on DNA conformation modification and subsequent identification on the location of the DNA damage which should be responsible for the mutation induction and DNA lesion characteristics. The results would enable us to understand how low-energy plasma ions induced mutations in DNA sequences, and could be applied to improve enzyme properties for low cost animal feed and fermentation industry. Moreover, plasma technology is cost-effective, and requires simple maintenance.

Experiments

1. Bacterial strain & Plasmid

GFP gene fragments were provided by Dr. Ruttaporn Chundet (Divison of Biotechnology, Faculty of Science, Maejo Universisty). The full length of *GFP* gene was amplified and then inserted into the corresponding sites (*BamH*I and *Hind*III) downstream of the *LacZ* operator as shown in Fig. 1. The plasmid DNA was amplified following transformation into *Escherichia coli* strain DH5α and subsequently extracted and purified using a GeneJET Plasmid Purification kit (Thermo scientific, Lithuania) according to the manufacturer's protocol. The plasmid DNA produced by this procedure was dissolved in sterile, elution buffer resulting in a plasmid concentration of 0.1 μg/μl.

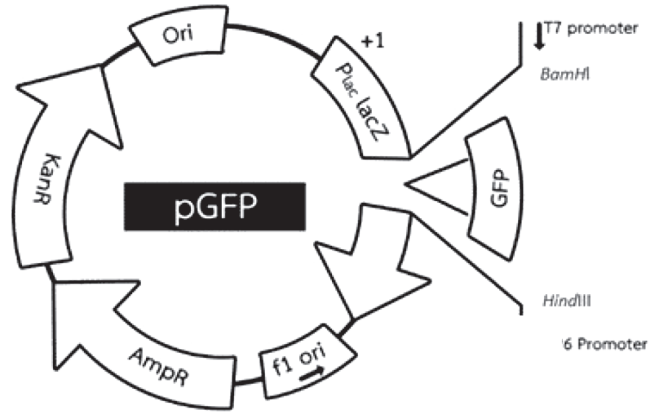

Figure 1 Map of pGFP which carries the pGFP-derived *Bam*HI replicon (f1 *ori* and *Ori*), *lacZ* gene, Ampicillin resistance gene (*AmpR*) and Kanamycin resistance gene (*KanR*)

Figure 2 DNA sample holder.

2. Plasma ion bombardment

Aliquots of 10 µl plasmid DNA solution were deposited in sample pots of a sample holder. The holder was made from stainless steel and had 9 holes on it with each in a size of 5 mm in diameter and 5 mm in depth, as shown in Fig. 2. A hole containing the control was covered by carbon tape. Plasma bombardment of the naked DNA samples was carried out using our in-house developed atmospheric pressure plasma jet (APPJ) at Biotechnology Unit, University of Phayao. In the device, the hollow quartz tube was used as dielectric barrier discharge. Plasma was produced by supplying 25 kHz high voltage pulsed DC power on solid HV electrode. Plasma then passes through a jet head to arrive on the surface of the material to be treated. A schematic of the APPJ system comprised of a high voltage power supply, electrodes and a dielectric tube was shown in Fig 3. Argon (Ar) and Helium (He) were used as the plasma source material for bombardment. The plasma was generated with energy of 100 watts for 10 and 15 min and flow rate at 2 sccm.

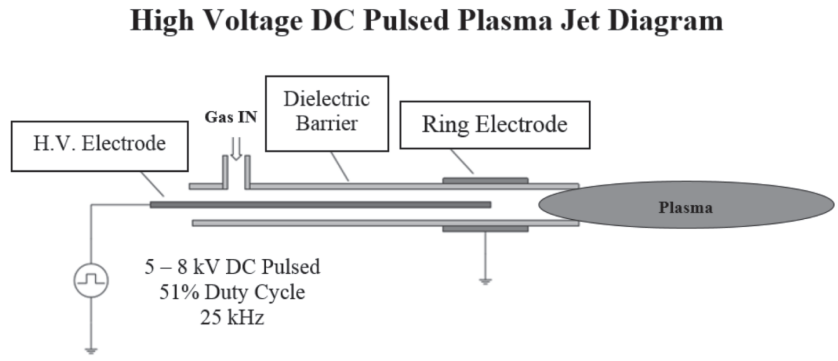

Figure 3 Schematic drawing of atmospheric pressure plasma jet (APPJ) device at Biotechnology Unit at University of Phayao.

3. Gene transformation, mutation analysis and DNA sequencing

After the plasma bombardment, the samples were recovered in 10 µl of de-ionized water and divided into two parts for analysis. The first part was gel electrophoresis to analyze DNA conformation including relaxed, supercoiled and linear bands. Sample aliquots of 5 µl were added with 1 µl of gel loading buffer and then loaded onto wells with 1.2% (w/v) agarose gel made up in TBE buffer. This gel was run at constant voltage (100 V cm^{-1}) for approximately 1 h. Images of the gels were captured using LED-transilluminator and digital camera. For the other part, the plasma treated plasmid was transformed into *E. coli* DH5α competent cells using the standard electroporation. DNA mutation screening was indicated by the white bacterial colonies under UV-transilluminator. White colonies were streaked in 5 generations on plates again to check for their stability of the phenotype. After that, DNA sequencing of the selected mutants and the control were performed by Macrogen, Korea. The DNA sequencing result was compared with the control pGFP sequence for analysis of the nucleotide modification. The mutation frequency of a certain nucleotide was calculated as a ratio between the number of the nucleotide modification and the total number of the changes in all nucleotides. The total changes of each nucleotide for each type of mutations was summarized and used to indicate the radiosensitivity of the nucleotide, which was defined as a ratio between the total changes of a nucleotide and the total number of the changes in all nucleotides starting from *LacZ* promoter to the stop codon of *GFP* gene.

Results

1. Effects of plasma bombardment of plasmid DNA on mutation

The DNA forms after plasma ions bombardment were analyzed with gel electrophoresis as shown in Fig. 4. The results clearly showed that the supercoiled form of plasmid decreases while the linear form increases as bombardment time was longer. The supercoiled form is the original DNA form and normally when a single strand break (SSB) or double strand break (DSB) occurs the supercoiled form was damaged (broken) into the linear form. Both of Ar and He plasmas caused certain SSBs and the highest intensity of linear form was observed under Ar plasma treatment for 15 min compared with other treatment times. This is a clear indication of the ion direct interaction with DNA responsible for the DNA strand breaks.

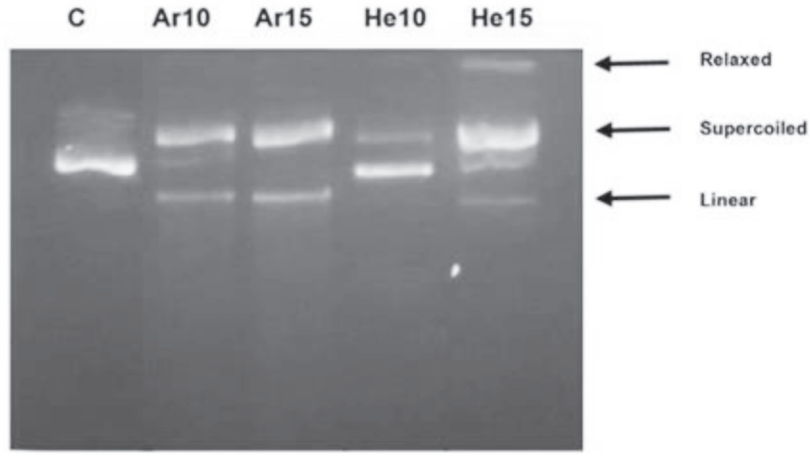

Figure 4 Electrophoretic profiles results. (a) Effects of Ar and He plasma bombardment on plasmid DNA, with energy of 100 watts for 10 and 15 min. Keys: C: control, Ar10: Ar plasma bombarded for 10 min, Ar15: Ar plasma bombarded for 15 min, He10: He plasma bombarded for 10 min, He15: He plasma bombarded for 15 min.

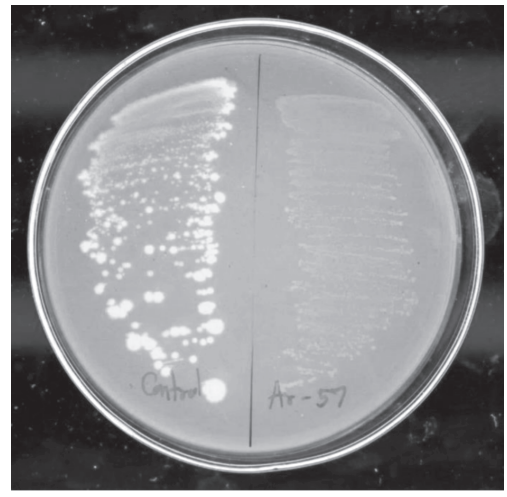

Figure 5 Observation of the Ar plasma bombardment on plasmid DNA in transformed *E. coli* (DH5α) under UV light. The restreaked bacterial green colony (left, control) compared with white colony (right).

2. Mutant screening

For the mutant screening, the treated plasmid DNA from each bombardment condition was transferred into *E. coli* cells. Results showed that white bacterial colonies were observed only under Ar plasma bombardment for 15 min (Fig. 5), whereas other bombarded conditions (Ar plasma bombardment for 10 min, He plasma bombardment for 10 and 15 min) generated only green fluorescence bacterial colonies. These white colonies were mutants because the transformed DNA inside the bacterial cells was destructed and thus the characteristic green fluorescence expression under UV light could not function. The white colonies were then picked out, cultured and plated again to check their purity, and hence the white colonies were indicated as the mutants. All 10 colonies of mutants which obtained from Ar plasma bombardment were selected, and plasmid DNA was extracted for DNA sequence analysis.

3. Sequence analysis

To reveal the modification of DNA which caused a phenotypic change due to plasma treatments, the DNA sequences of 10 mutant clones from the Ar plasma bombardment including *LacZ* operator, T7 promoter and *GFP* gene were compared with the control using Clustal Omega program, as shown in Fig. 6. However, among 10 mutants (A1-A10), only 2 mutants named A- 7 and A-9 showed nucleotide modification in DNA sequence alignment. The nucleotide sequence modification was summarized in Table 1. The results showed that both substitution and insertion mutations were observed under Ar plasma bombardment with different frequencies,

whereas nucleotide deletion was not detected in this experiment. The nucleotide insertion was dominant with high frequencies, while nucleotide substitution including transversion and transition, were minor with low frequencies. In transversion, G→T had the highest frequency (27.8%), about seven folds greater than both G→C and T→A, which showed the lowest frequency (3.9%). In addition, nucleotide changing from both T→G and A→T had equal frequency of 13.2%, while the changing of both C→A and C→G showed 9.2%. For transition, the highest frequency was found for the change from G→A (11.8%), whereas the changing from C→T had the lowest frequency (2.6%).

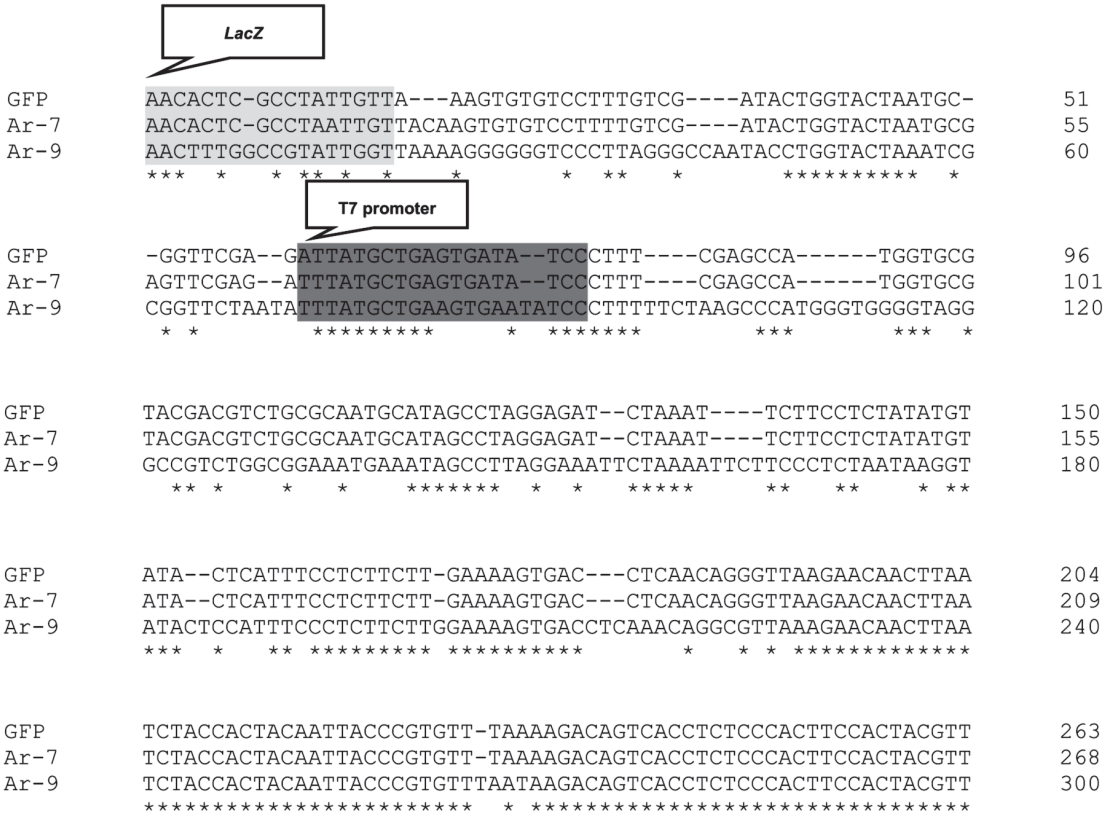

Figure 6 Comparison of the base sequence between pGFP (control) with mutant clones, A-7 and A-9, from the Ar plasma bombardment for 15 min. The stars indicated identical bases. The DNA sequencing analysis revealed the base insertions and substitutions.

Table 1 Observed types and numbers of occurrence of mutations in the mutant clones from the Ar plasma bombardment.

Mutation type	Number of occurrence		Frequency (%)	
	(a) A-7	(b) A-9	(a) A-7	(b) A-9
Base substitution	18	76	100.0	100.0
Transition	7	22	38.9	28.9
A→G	2	5	11.1	6.6
G→A	2	9	11.1	11.8
C→T	1	2	5.6	2.6
T→C	2	6	11.1	7.9
Transveraion	11	54	61.1	71.1
C→A		7		9.2
T→G	3	10	16.7	13.2
A→T	2	10	11.1	13.2
C→G	1	7	5.6	9.2
G→T	5	5	27.8	6.6
A→C		3		3.9
G→C		3		3.9
T→A		9		11.8
Insertion	5	37	27.8	48.7
+A	3	7	16.7	9.2
+G	1	7	5.6	9.2
+C	1	8	5.6	10.5
+T		15		19.7
Total number of occurrences	18	76	100	100

Conclusions and Discussion

The result of gel electrophoresis indicated that Ar plasma bombardment for 15 min created damage to the plasmid DNA and caused malfunction of the DNA resulting in white color expression of the bacterial cells after plasmid transformation. DNA conformation modifications induced by the low-energy plasma bombardment included changes from the natural supercoil to relaxed form and even linear form due to either single or double strand breaks [9]. As bombardment time of plasma ions is directly related to the DNA damage, the result demonstrated that the longer time of the ion bombardment, the higher the DNA strand breaks were observed. In Fig. 4, the results clearly showed that the intensity of the relaxed form of the plasmid was increased when the longer time of plasma treated. This indicated that the supercoil DNA was damaged and changed to relaxed form upon Ar plasma bombardment for 15 min. This result was evident that the direct interaction of ions to DNA structure was responsible for the DNA strand breaks. These DNA strand breaks could be repaired wrongly after the plasmid was transferred in to the bacteria resulting in mutation. It should be noticed that in our plasma treatments of DNA the linear form of DNA appeared negligibly compared to the experiment on ion beam bombardment of DNA. This was due to the different energy levels applied [10]. However, the mutation rate with a very low frequency was observed in the bacterial mutants bombarded by Ar plasma for 15 min. Regarding ion plasma bombardment, the higher of ion energy or larger of molecular weight, the more the radiation damage was observed [11]. Molecular weight of Ar is ten folds higher than that of He (molecular weight of Ar and He are 40 and 4, respectively) [12]. The electron density of the Ar plasma ions is twice larger than He plasma ions at the same applied voltage [13]. These data could indicate that the Ar plasma has more effect to DNA damage than that of the He plasma. So, we could observe the bacterial mutants only under 15 min of Ar plasma bombardment. This result further explained the direct ion interaction with DNA causing the DNA breaks and leading to mutations. The plasmid pGFP has three main components as shown in Fig. 1. When plasmas bombarded the whole DNA, the plasmas were distributed to every component, and hence the *GFP* gene was bombarded randomly as shown in Fig. 6. The nucleotide modifications not only observed in *GFP* gene, but also observed in *LacZ* operator and T7 promoter as well resulting in white bacterial colonies. This result indicated that Ar plasma bombardment was random mutation to naked plasmid DNA. In addition, it is seen that the nucleotide insertion was a dominant type of mutation. Although 10 white colonies were found only two colonies showed bases modification in this experiment. In conclusion, Ar plasma bombardment was successfully induce mutation in naked DNA, it is suggested that plasma technology could be applied for bacterial enzyme property improvement at DNA level in future research.

Acknowledgments

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